# NANOSECOND SEGMENTAL MOBILITIES OF TRYPTOPHAN RESIDUES IN PROTEINS OBSERVED BY LIFETIME-RESOLVED FLUORESCENCE ANISOTROPIES

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ABSTRACT Steady-state and lifetime-resolved fluorescence anisotropy measurements of protein fluorescence were used to investigate the depolarizing motions of tryptophan residues in proteins. Lifetime resolution was achieved by oxygen quenching. The proteins investigated were carbonic anhydrase, carboxypeptidase A, α-chymotrypsin, trypsin, pepsin, and bovine and human serum albumin. When corrected for overall protein rotation, the steady state anisotropies indicate that, on the average, the tryptophan residues in these proteins rotate 29° ± 6° during the unquenched excited state lifetimes of these proteins, which range from 1.7 to 6.1 ns. The lifetime-resolved anisotropies reveal correlation times for these displacements ranging from 1 to 12 ns. On the average these correlation times are tenfold shorter than that expected for overall protein rotation. We conclude that the tryptophan residues in these proteins display remarkable freedom of motion within the protein matrix, which implies that these matrices are highly flexible on the nanosecond time scale.

## INTRODUCTION

Recent fluorescence spectroscopic studies have revealed structural mobility in proteins which occurs on the nanosecond time scale. These observations include rapid diffusion of quenchers through the matrices of many proteins (Lakowicz and Weber, 1973*b*; Eftink and Ghiron, 1976), dipolar relaxation around the excited states of tryptophan residues and other fluorophores (Grinvald and Steinberg, 1974; Brand and Gohlke, 1971; Lakowicz and Cherek, 1980) and rapid tryptophan rotations as revealed by time-resolved decays of fluorescence anisotropy (Munro et al., 1979). In this investigation we use oxygen quenching of protein fluorescence to vary the lifetime of the excited state. Steady-state anisotropy measurements under these quenching conditions can reveal the rotational correlation times of the fluorophores (Lakowicz et al., 1979), which in this case are the tryptophan residues in proteins.

An advantage of these lifetime-resolved measurements is that they can be performed without significant variation of solvent viscosity or temperature. Such variation can alter the extent to which depolarization results from overall protein rotation and from segmental motions of the tryptophan residues or domains in the protein (Weltman and Edelman, 1967; Wahl and Weber, 1967). Moreover, changes in temperature or solvent composition can alter the relative energy levels of the $^1L_a$ and $^1L_b$ transitions, leading to changes in $r_0$, the limiting anisotropy.

## THEORY

The average angular displacement ($\theta$) of a spherical molecule in a fixed time interval may be characterized by the quantity $d$ (Soleillet, 1929).

$$d = \langle 3 \cos^2 \theta - 1 \rangle / 2. \quad (1)$$

As shown by Soleillet the total angular effect produced by two independent causes acting on a freely rotating molecule is given by the product

$$d = d_t \, d_p. \quad (2)$$

In our case $d_t$ and $d_p$ correspond to effects brought about by rotation of the tryptophan residues within the protein and rotations of the whole protein, respectively. The fluorescence depolarization owing to the motion is given by the simple expression

$$r = r_0 \, d, \quad (3)$$

where $r$ is the observed anisotropy and $r_0$ is the anisotropy in the absence of rotation. Equations 1–3 can be applied to our case only as a first and very coarse approximation. The motion of the tryptophan residues independent of the protein is a complex hindered motion, and cannot be considered as producing effects that are of the same nature as those of the free rotation of the protein as a whole. However, we are not interested here in the fine details of the motions, but simply in calculating an overall angular displacement of the tryptophan residues in relation to the rest of the molecule. For such a purpose Eqs. 1–3 are sufficient, and Eq. 3 permits determination of the overall Soleillet factor $d$. Moreover, in Eq. 2, $d_p$, the angular motion of the protein as a whole, may be estimated by the use of the Perrin equation (1936),

$$d_p^{-1} = 1 + \tau/\phi_p, \quad (4)$$

where $\tau$ is the fluorescence lifetime and $\phi_p$ is the rotational correlation time (equal to one-third of the rotational relaxation time of Debye). To a good approximation globular molecules obey the relation

$$\phi_p = \alpha(\eta V/RT), \quad (5)$$

where $\eta$ is the viscosity of the medium; $V$, the molecular volume; $R$, the gas constant; $T$, the absolute temperature; and $\alpha$, a form and hydration factor which is usually close to 2.

Similiarly, we can set

$$d_t^{-1} = 1 + \tau/\phi_t \quad (6)$$

which provides us with a means of determining an effective or average correlation time of the independent tryptophan motions ($\phi_t$).

In this case the magnitude $d_t^{-1}$ or $\phi_t$ was estimated from the variation in fluorescence anisotropy as the lifetime is varied by oxygen quenching. We acknowledge that the apparent correlation time ($\phi_A$) so derived (see Methods) does not have the physical significance of a free rotational motion, but rather describes the speed of restricted motion during the time scale of the fluorescence lifetime.

## METHODS

Quenching of protein fluorescence by oxygen requires equilibrium of the aqueous protein solution with oxygen pressures ranging to 100 atm, as described previously (Lakowicz and Weber, 1973*a*). For these studies the pressure cell was mounted in a T-format fluorescence polarization instrument (Weber and Bablouzian, 1966) so that the horizontal and vertical components of the emission could be observed simultaneously. We found that the long quartz windows used for our earlier quenching studies (Lakowicz and Weber, 1973*a*) caused depolarization, presumably as a result of optical flaws present in

the quartz. This effect was avoided by using 0.25-in thick fused quartz windows. The sample was contained in a 2 × 2-cm cuvette, 4 cm high, which was contained in the pressure cell. Reflected light within the cell was minimized with a Corning 2–64 filter (Corning Glass Works, Science Products Div., Corning, N. Y.) positioned to absorb exciting light after it had passed through the sample. The excitation bandpass was 3.3 nm. A Corning 7–54 filter was used in the exciting beam to reduce stray light and the emission was observed through Corning 0–52 filters. Emission spectra taken under identical optical conditions indicated that scattered or stray light did not interfere with any of our measurements, even under conditions of maximum quenching. Moreover, the excitation polarization spectra obtained using the pressure cell were identical to those obtained using the more common 1 × 1-cm cuvettes, both for the proteins at 25°C and for *N*-acetyl-L-trytophanamide (NATA) at -62°C.

The sources of proteins and other materials have been described earlier (Lakowicz and Weber, 1973*b*). Identical buffers were also used for each protein. Unless otherwise indicated, all measurements were performed at 25 ± 0.2°C. The optical densities of NATA and the protein solutions ranged from 0.1 to 0.3 at 280 nm.

The fluorescence lifetimes ($\tau$) for the proteins at each oxygen pressure were calculated from the Stern-Volmer equation

$$\tau = \tau_0/[1 + K(0_2)], \tag{7}$$

using the unquenched lifetimes ($\tau_0$) and quenching constants determined previously (Lakowicz and Weber, 1973*b*). For NATA in propylene glycol the lifetimes were calculated from the dynamic portion of the observed quenching (Lakowicz et al., 1979) which was found to be 19.6 $M^{-1}$ using an oxygen solubility of 0.00143 M/atm (Osborn and Porter, 1964). (This solubility was obtained from Fig. 3 in the latter reference, and appears to differ from the value reported in the text.) In contrast to aqueous solutions which require 30 min for oxygen equilibration, propylene glycol solutions require at least 5 h with continuous stirring.

We fit our lifetime-resolved anisotropies to

$$r = r_\infty + (r_0 - r_\infty)d_A \tag{8}$$

where $r_\infty$ is the anisotropy which would be observed at times which are much longer than the fluorescence lifetime, and $d_A$ is related to an apparent correlation time $\phi_A$, as indicated in Eqs. 4 and 6. Apparent $\phi_A$ values which are shorter than that expected for overall protein rotation ($\phi_p$) are taken to indicate internal tryptophan rotations. At present we attach no physical significance to the $r_\infty$ values, except for the improved fit of our data. Munro et al. (1979) did observe hindered rotations of tryptophan in human serum albumin (HSA) and azurin, and such motions could result in apparent nonzero $r_\infty$ values. However, these nonzero $r_\infty$ values we observe are equally likely to be a result of the nature of lifetime-resolved anisotropy measurements, which must necessarily be obtained for times equal to or shorter than the unquenched lifetimes. As a result, these measurements are most sensitive to the more rapid motions, but the time scales of the slower overall protein rotations becomes unobservable. These slower motions are revealed only by an apparent nonzero $r_\infty$ value.

## RESULTS

Steady-state fluorescence anisotropies were measured under quenching conditons for the proteins listed in Table I and for NATA in propylene glycol at 25°C. Representative data for NATA and pepsin are shown in Fig. 1. As expected, these anisotropies increased with decreasing lifetime since these conditions allow less time for reorientations of the tryptophan residues. We choose to use the anisotropies obtained at 300 nm excitation, although similar results were obtained using excitation wavelengths from 295 to 310 nm. Energy transfer between tryptophan residues would result in depolarization of the emission (Weber, 1966) and consequently shorter apparent correlation times. However, such transfer is unlikely at 300 nm excitation (Weber, 1960; Weber and Shinitzky, 1970). Moreover, at 300 nm one excites primarily the $^1L_a$ state of tryptophan (Valeur and Weber, 1977).

TABLE I
AVERAGE ANGULAR DISPLACEMENT OF TRYPTOPHAN RESIDUES IN PROTEINS IN EXCESS OF OVERALL ROTATIONAL DIFFUSION

| Protein | $\tau_o$ | $\phi_p$* | $\theta$‡ |
|---|---|---|---|
| | (*ns*) | (*ns*) | |
| Pepsin | 5.5 | 19 | 29 |
| Carboxypeptidase A | 1.7 | 19 | 36 |
| α-chymotrypsin | 2.1 | 13 | 31 |
| Human serum albumin | 6.0 | 33 | 19 |
| Bovine serum albumin | 6.1 | 33 | 22 |
| Trypsin | 1.9 | 13 | 27 |
| Carbonic anhydrase | 4.5 | 17 | 36 |

*$\phi_p = \eta v/kT \times 2$.
‡From Eqs. 1–3.

Assuming the $r_0$ value for NATA also applies to the tryptophan residues in the proteins, we calculated the average angular displacements ($\theta$) of these residues due to segmental mobility, as is illustrated in Fig. 2 *b* for pepsin. We note that $\theta$ is obtained from the observed anisotropy, which is actually a measure of $\cos^2\theta$. Among the proteins listed in Table I, pepsin has the highest ratio of $\phi_p$ to $\tau_o$, and hence the largest amount of depolarization due to rotational diffusion. The extent of depolarization expected as a result of such diffusion is shown by the solid line in Fig. 2 *B*. Clearly, the observed anisotropy of emission at $\tau = 5.5$ ns (unquenched) is significantly lower than predicted for a protein with rigidly fixed tryptophan residues. We used Eqs. 1–5 to calculate the average angular displacement due to segmental mobility, which for pepsin is 29°. Similarly we used the unquenched anisotropies to calculate $\phi$ for the other proteins. The extent of depolarization resulting from segmental mobility is indicated by the arrow on Fig. 3, and the average angular displacements are summarized in Table I. For the seven proteins investigated these displacements average 29° ± 6°. We conclude that the steady-state anisotropies indicate significant internal mobility of the tryptophan residues in

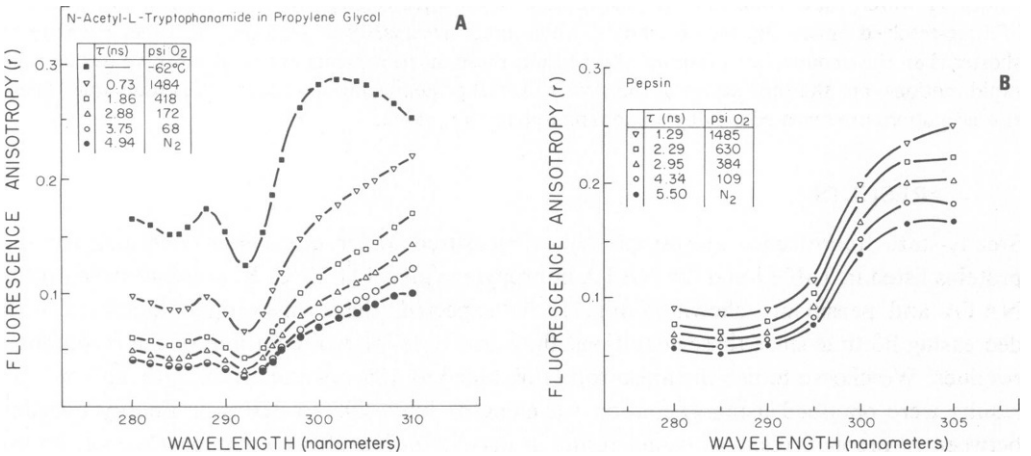


Figure 1 Lifetime-resolved fluorescence anisotropies. Excitation polarization spectra are shown for NATA in propylene glycol and for pepsin. Lifetimes were calculated from Eq. using the quenching constants listed in Lakowicz and Weber (1973*b*).

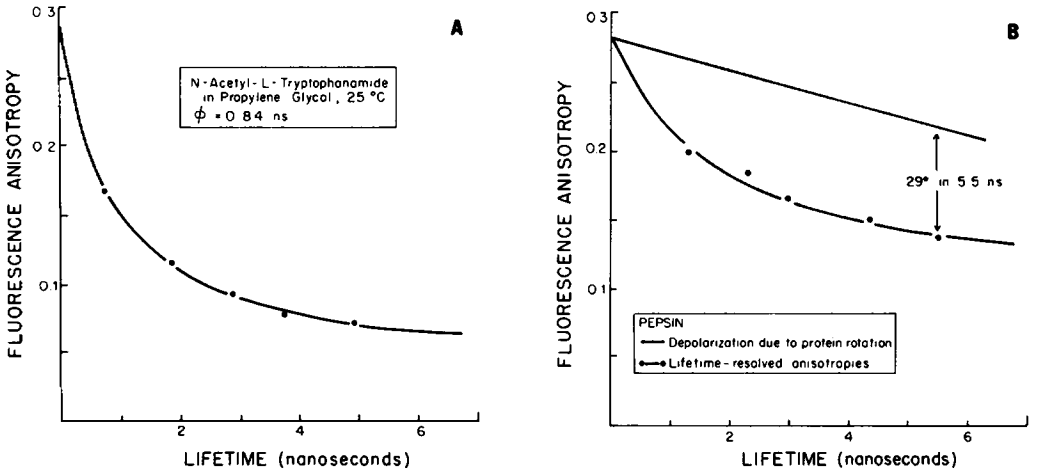


Figure 2 Lifetime-resolved anisotropies for NATA in propylene glycol (*A*) and pepsin in aqueous buffer (B). The anisotropies are for excitation at 300 nm. The line through the data points is the theoretical curve for the $\phi_A$ and $r_\infty$ values listed in Table II. In 2B the solid line shows the depolarization expected for overall protein rotational diffusion.

these proteins which result in average displacements of 30° during the 2–6-ns lifetimes of their excited states.

To determine the time scale of these angular displacements we used the lifetime-resolved anisotropies. Fig. 2 *A* shows lifetime-resolved anisotropies for NATA in propylene glycol at 25°C, and the theoretical curve which best fits these data. The observed correlation time is 0.84 ns, which is about threefold smaller than that calculated from Eq. 5, which is 2.7 ns. (The volume was assumed to be 200 ml/mol). Small molecules frequently rotate more rapidly than predicted from the Stokes-Einstein equation (Mantulin and Weber, 1977). We note that

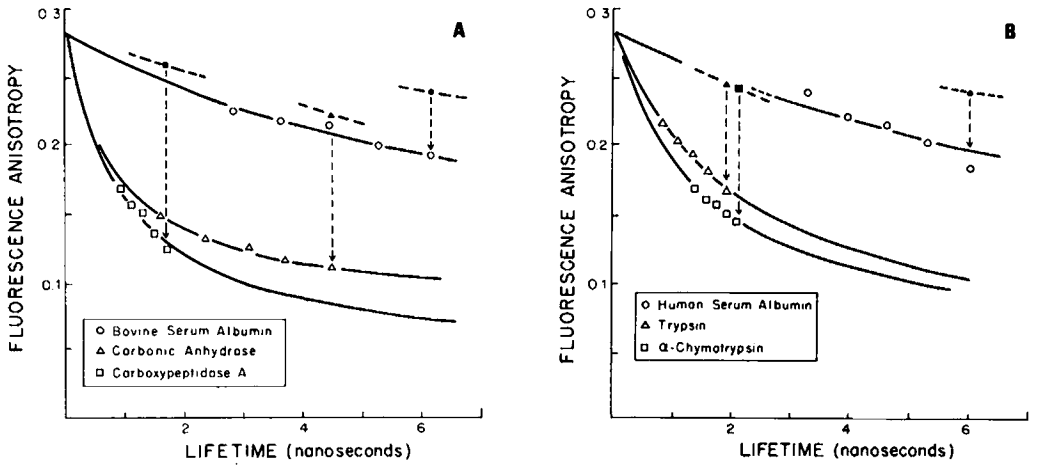


Figure 3 Lifetime-resolved anisotropies of proteins. In each case the open symbols represent the data, and the corresponding closed symbols illustrate the amounts of depolarization expected for rotational diffusion of the proteins. The dashed line and arrows illustrate the extent of depolarization found in excess of that expected for a rigid body of equivalent molecular weight and lifetime.

TABLE II
AVERAGE CORRELATION TIMES OF TRYPTOPHAN RESIDUES IN PROTEINS

| Sample | $\phi_A$* | $r_\infty$* | $\phi_p/\phi_A$* | $r_0$‡ | $\phi_A$‡ | $\phi_p/\phi_A$‡ |
|---|---|---|---|---|---|---|
| | (*ns*) | | | | (*ns*) | |
| NATA in propylene glycol | 0.84 | 0.036 | – | 0.244 | 1.6 | – |
| Pepsin | 1.75 | 0.095 | 11 | 0.235 | 8.0 | 2.4 |
| Carboxypeptidase A | 1.03 | 0.038 | 18 | 0.290 | 1.3 | 15 |
| α-chymotrypsin | 1.50 | 0.047 | 9 | 0.244 | 3.0 | 4.3 |
| Human serum albumin | 12.0 | 0.03 | 2.8 | 0.299 | 7 | 4.7 |
| Bovine serum albumin | 9.0 | 0.06 | 3.7 | 0.270 | 16 | 2.1 |
| Trypsin | 2.3 | 0.035 | 5.8 | 0.278 | 2.9 | 4.5 |
| Carbonic anhydrase | 0.83 | 0.079 | 20 | 0.180 | 7 | 2.4 |

*From fit of data to Eq. 6. The correlation times obtained using $r_\infty$=o were similar to those listed above, but the fits of the lifetime-resolved anisotropies to the theoretical curves were substantially poorer.
‡Obtained by application of Eq. 9, as illustrated in Fig. 4.

the apparent $r_\infty$ value for NATA is similar to those found for the proteins (Table II) and that at present we attach no significance to these values except for the improved fit to the data.

We also obtained the apparent correlation times of the tryptophan residues from lifetime-resolved anisotropies. These data and theoretical curves are shown in Figs. 2 *B*, and 3, and the correlation times are summarized in Table II. The apparent correlation times range from 0.83 ns for carbonic anhydrase to 12 ns for HSA. The latter value is intermediate between the correlation times observed by Munro et al. (1979) for HSA at 8 and 43°C, and thus may be considered to be in agreement with their results. Moreover, $\phi_A$ values as short as 0.09–1.26 ns were observed by these workers for basic A1 myelin protein, and hence our 0.83 ns value for carbonic anhydrase is not without precedent.

Remarkably, all the apparent correlation times for tryptophan rotation are shorter than those expected for rotational diffusion of the proteins. The ratio $\phi_p/\phi_A$ varies from 3 to 20 for the seven proteins investigated, the average ratio being 10 ± 7. We conclude that our lifetime-resolved anisotropy data reveal tryptophan displacements which are, on the average, ten-fold more rapid than protein rotation. Such displacements again indicate the highly dynamic nature of protein matrices on the nanosecond time scale.

We also analyzed our results by direct application of the Perrin equation

$$1/r = {}^1/r_0 + (\tau/r_0\phi_A), \tag{9}$$

using plots of $1/r$ versus $\tau$. In this type of analysis the motions which are very fast compared with the shortest of the achieved lifetimes will make the $r_0$ value obtained by extrapolation to $\tau$ = o inferior to the $r_0$ value for a truly motionless residue (NATA in propylene glycol at -62°C). On the other hand the value of $\phi_A$ calculated from Eq. 9 will exceed the value calculated using the motionless $r_0$ value.

For NATA in propylene glycol a plot of $1/r$ versus $\tau$ yields $r_0 = 0.244$ and $\phi_A = 1.6$ ns (Fig. 4 *A* and Table II). In comparison $r_0$ at -62°C was observed to be 0.2824, and the previous method of data analysis yielded $\phi_A = 0.84$ ns.

The data for proteins were analyzed similarly (Fig. 4 and Table II). It is interesting to note that all the intercepts (with the exception of carbonic anhydrase) cluster around that observed for NATA (0.269 ± 0.025). This observation supports our choice of the $r_0$ values from NATA at -62°C in our earlier analysis. Carbonic anhydrase is an exception, with the intercept

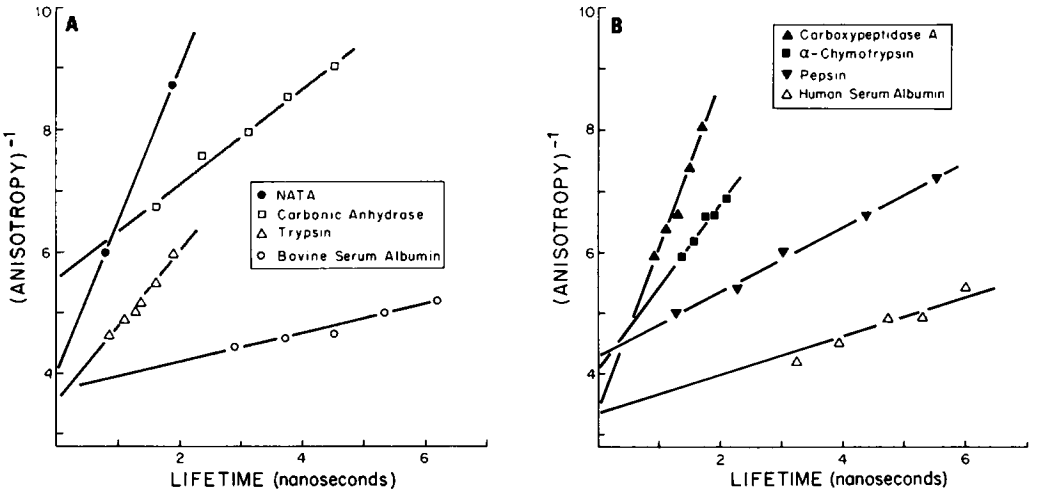


Figure 4 Perrin-lifetime plots.

yielding a lower $r_0$ value (0.180). We suspect this apparently low $r_0$ value to be a result of tryptophan rotations which occur on the subnanosecond time scale, and which are not apparent at even the shortest quenched lifetime (1.6 ns).

The apparent correlation times derived by this method are also summarized in Table II. On the average these values are twofold longer than those derived from Figs. 2 and 3, but the ratio of $\phi_p/\phi_A$ is still 5 on the average. Thus, whether one chooses to maximize or minimize $\phi_A$, the data reveal the existence of tryptophan displacements which are too rapid to result from rotation of the protein as a whole.

## DISCUSSION

With the exception of human serum albumin, the proteins investigated all contain more than a single tryptophan residue. Thus, the observed fluorescence anisotropies result from a hetergeneous population of fluorophores. To evaluate the effect of such heterogeneity on our lifetime-resolved anisotropies we present one simple model in which we assume that two types of tryptophans exist which differ only in their correlation times $\phi_1$ and $\phi_2$. The effects of heterogeneity will be maximal when each population contributes 50% to the total fluorescence intensity ($f_1 = f_2 = 0.5$); we assumed these proportions in our calculations.

Our early studies of oxygen quenching of tryptophan residues in a variety of proteins revealed that the quenching constants and lifetimes differed by no more than a factor of 4 (Lakowicz and Weber, 1973*b*). On the other hand the studies of Munro et al. (1979) showed that the correlation times can differ by a factor of 350 (31.4 ns for HSA and 0.09 ns for basic A1 myelin protein). Thus we can assume that the difference in $\tau_0/\phi$ essentially reflects differences in the correlation times. We therefore assumed $\tau_0$ to be constant in our simulation (5 ns).

The observed fluorescence anisotropy ($r_{obs}$) resulting from two populations of fluorophores is given by

$$r_{obs} = r_1 f_1 + r_2 f_2 \tag{10}$$

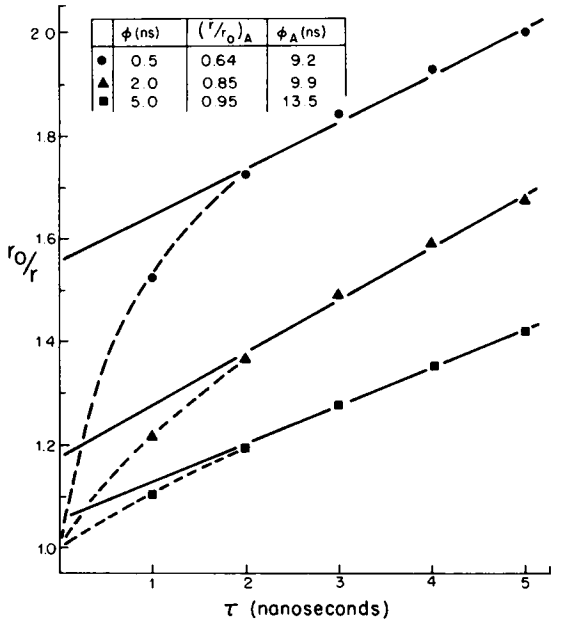


Figure 5 Theoretical Perrin-lifetime plots for a heterogeneous fluorophore population. The lifetimes and fractional intensities of both populations are assumed equal. The slower correlation time ($\phi_2$) is assumed constant and equal to 50 ns. The dashed lines represent the complete curve and the symbols represent illustrated data points. The solid lines are drawn through the theoretical data points for lifetimes in excess of 2 ns, which is typical of the range of lifetimes available using our experimental conditions.

where $r_i$ and $f_i$ are the anisotropies and fractional intensities of each population. The individual anisotropies are given by

$$r_i = r_0/(1 + \tau/\phi_i) \tag{11}$$

As a particular example we present theoretical plots (Fig. 5) in which we allow one of the populations to rotate with increasing rapidity ($\phi_1 = 5, 2, 0.5$ ns) while the second population is restrained to rotate with the protein as a whole ($\phi_2 = 50$ ns).

Recall that lifetime-resolved anisotropies can be obtained only over a limited range of lifetimes. On the average, a protein with an initial lifetime of 5 ns could be quenched to a minimum lifetime of 2 ns using our experimental conditions. Under this constraint two points are worthy of mention. First, the limiting anisotropy, obtained by extrapolation to $\tau = 0$, becomes increasingly smaller as one population rotates more freely. For example, $r/r_0$ at $\tau = 0$ decreases to 0.64 as $\phi_1$ is decreased to 0.5 ns. Such a freely rotating population may be responsible for the low $r_0$ value for carbonic anhydrase which was observed by extrapolation. Second, such heterogeneity is unlikely to give rise to observable curvature in the Perrin-lifetime plots, but would be revealed by an apparent correlation time which is shorter than that due to overall protein rotations. For example, the existence of a rapidly rotating population yields apparent correlation times near 12 ns. Recall that on the average, the apparent correlation times we calculated from the Perrin-lifetime plots were fivefold smaller than that expected for overall protein rotation. We conclude that at least a significant fraction of the tryptophan residues in these proteins have considerable segmental mobility, but that our observations do not exclude the existence of regions which are relatively immobile in times of the order of $\tau_0$ and which rotate only as a result of overall protein rotation.

In summary, for the seven proteins investigated, the lifetime-resolved anisotropies obtained under quenching conditions indicate considerable angular displacements ($\simeq$30°) of tryptophan residues which occur on the nanosecond time scale. The precise magnitudes of these displacements, and their correlation times, are somewhat uncertain as a result of heterogeneity in the emitting population and the limited range of lifetimes observable by quenching. However, regardless of our mode of data analysis, it is clear that these displacements occur and must occur in a significant proportion of the trypotophan residues. In total, these observations, along with those of other researchers (Brand and Cohlke, 1971; Grinvald and Steinbeg, 1974; Munro et al., 1979) and the computational studies of Karplus and co-workers (McCammon et al., 1977) reveal that proteins contain many degrees of freedom for displacements of individual residues or regions. The biological significance of these motions, and their relationship to enzymic activity, remain to be elucidated.

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## DISCUSSION

*Session Chairman*: Hans Frauenfelder *Scribe*: William A. Wegener

BRAND: I'd like to emphasize that one measures rotations of the emission dipole and not necessarily of the whole molecule. You have indicated some evidence for solution interactions. If these interactions occur on a nanosecond timescale, they might cause a loss of anisotropy which has nothing to do with tryptophen rotations.

LAKOWICZ: A solvation dependent $r_0$ requires that the solvent change the dipole orientation in the molecule itself. I know of no such example, and with regard to polar solvents, I think Stryer's data argue against it. He looks at an indole derivative with increasing viscosities, which slows molecular rotations. When he extrapolates his data to time zero, in each case he obtains the same $r_0$ value.

ROSS: We have used pulse methods to look at the two tryptophans in liver alcohol dehydrogenase. The average lifetime across the emission spectrum does not necessarily imply solvent relaxation. We found that you can separate the contribution of the two residues since one is blue-shifted and the other is red-shifted. The life-times do not vary with wavelength but the weighting does. Thus, if you calculate the average lifetime, the average increase may not necessarily have to do with solvent relaxation. That is, you may need more information than you get from the phase measurement.

LAKOWICZ: We did take a chance with a two tryptophan protein and hope that we selected the internal residue. You may be right, and if we've made a mistake, I'll be pleased to retract it.

HARVEY: Your model is consistent with your data, but there may be another effect besides tryptophan motion which arises from several lifetimes. From your equation, the observed anisotropy is a weighted average of the different fluorophores present. As a simple example, consider two fluorophores with different single lifetimes which do not move. The anisotropy will show a time-dependence. What evidence do you have that this type of mechanism does not account for your polarization decays?

LAKOWICZ: On a probability basis, it is unlikely that this would occur for the seven proteins and others we've looked at. With regard to heterogeneity, all the proteins except HSA are multi-tryptophan proteins. There can be heterogeneity in lifetimes, quenching constants and correlation times of the tryptophan moieties. I'll argue that the dominant effect is heterogeneity in correlation times. Quenching is relatively nonselective. We see no relationship between quenching constants and the tryptophan location, and we don't find we removed one group of tryptophans. Since our pepsin data indicated that lifetimes and yield decreased similarly, the fractional quenching could not be more than fourfold different between all tryptophans in the protein. Stryer's data indicate that the correlation time for tryptophans in a protein can vary by 350 or so. To model that, one can plot the limiting anisotropy divided by the anisotropy versus lifetime using two equal populations of fluorophores, one with a 50 ns correlation time equivalent to protein rotations, the others with a fast correlation time, either 5, 2 or 0.5 ns, attributed to internal mobility. Given the limitations of the lifetimes over which we can observe anisotropy, these mixed populations give a correlation time of ~10 ns; they do not select for the most rapid motions, but instead select for the slower motions. This effect could increase the observed correlation times several fold, but still leave them well below rigid protein rotational times. Thus, heterogeneity might shift our reported correlation times somewhat, but not our general conclusions about internal mobility.

LEHRER: How would any of your conclusions based upon oxygen quenching be modified for a charged quencher like iodide?

LAKOWICZ: As you know, iodide quenches much less effectively. Since it never makes it to the inside, one can separate internal and external tryptophans. We used oxygen here simply to vary the lifetimes.

SCHUSTER: What implications for oxygen-carrying molecules do the results that show oxygen diffuses into a protein have? Should we consider many different pathways for oxygen to reach the hemoglobin or myoglobin active site? Should the notion of a single set of activation barriers be refined?

LAKOWICZ: We have tried to block the pathways by which oxygen approaches the tryptophans. We buried the active site tryptophans of lysozyme without significant effect on oxygen's ability to reach them, which indicates there is no single energetic barriers. The activation energies for oxygen diffusion through proteins are small and ~4 kcal/mol. I'd like to ask Hans Frauenfelder whether our bodies would care if we slowed down the on/off rates of oxygen for hemoglobin by $10^3$–$10^4$, but kept the same binding parameters?

FRAUENFELDER: Yes they would. However, it is too late to begin a discussion of this sort. Most likely there is not one path, but then it is not like going through a sieve to reach the active site.

LAKOWICZ: While there are good reasons to believe protein thermal stabilities are generally related to decreasing dynamic properties, I would like to present one exception. We looked at the oxygen quenching of avidin in the presence and absence of biotin. Biotin greatly stabilizes avidin against denaturation, increasing its heat of denaturation from 70–270 kcal/mol. We expected this would greatly decrease the oxygen permeation rate through the protein, yet it diffuses through the more stable avidin-biotin complex about twice as rapidly as through the less stable avidin itself.

PRENDERGAST: We have done oxygen quenching studies on a number of proteins. With regard to the question of apomyoglobin pathways, we studied a number of fluorophores complexed to myoglobin and apomyoglobin. It appears that the activation energy is the same in apomyoglobin as in most proteins, both in terms of oxygen quenching tryptophan and quenching a fluorophore bound in the binding pocket. The curve for oxygen quenching shows a marked upward concavity, which indicates pseudostatic quenching. By that, I mean there is a finite amount of oxygen binding in the pocket which influences the diffusion rate of oxygen to the chromophore. This implies that there is no preferred pathway to the chromophore, but that there is a preferred binding region which influences the potential energy pathway for the process.